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14. ABSTRACT

TGF-β signaling represents a major tumor suppressor pathway. Loss of the TGF-β response is a hallmark in human cancer. However, the mechanisms underlying TGF-β resistance in breast cancer have not been elucidated. Anaplastic Lymphoma Kinase (ALK) is a tyrosine receptor kinase of insulin superfamily. IBC is relatively rare but the most lethal subtype of breast cancer. Thus, it is important to identify biomarkers, understand better current therapies and find new potential therapies for IBC. Our long-term goal is to understand the mechanisms underlying TGF-β resistance in human cancer. The short-term strategy of our research is to focus on ALK-induced inactivation of Smad4 in breast cancer. Our unifying **hypothesis** is that ALK causes TGF-β resistance through Smad4 inactivation and disrupts the growth constraints exerted by TGF-β signaling to promote breast tumorigenesis. To test our hypothesis, we propose the following specific aims to achieve our goals: 1. Investigate *in vivo* and clinical relevance of Smad4 tyrosine phosphorylation in breast cancer; 2. Determine the role of ALK-mediated Smad4 phosphorylation in TGF-β resistance in IBC; 3. Elucidate the molecular mechanisms underlying Smad4 tyrosine phosphorylation. This proposal will contribute significantly to breast cancer prevention and treatment.

15. SUBJECT TERMS
Smad4, ALK, breast cancer

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INTRODUCTION:

TGF-β exerts its tumor suppressing function by inhibiting the growth of normal epithelial cells. Loss of the TGF-β antiproliferative response is a hallmark in human cancers [1-3]. In TGF-β signaling pathway, tumor suppressor Smad4 plays a central role in TGF-β actions. Smad4 is frequently mutated or deleted in gastrointestinal and pancreatic cancer, which counts for TGF-β resistance in these cancers. However, not all types of cancers harbor deletion or mutations in the Smad4 gene. Inactivating mutations in the Smad4 gene are rare in breast cancers [4], but TGF-β response is attenuated [5,6], indicating that the tumor suppressor activity of Smad4 is abrogated by other mechanisms.

ALK is a tyrosine receptor kinase. Abnormal expression of ALK has been reported in numerous tumors including a significant fraction of breast cancer especially triple-negative breast cancer and inflammatory breast cancer [7]. ALK activation triggers major signaling pathways (MEK/ERK, STAT3, PI3K/Akt), which promote cell proliferation while preventing cell death [8-19]. However, the effect of ALK on TGF-β action, a major anti-proliferation function in cell, has not been explored.

In our preliminary studies, we have for the first time discovered that ALK could inactivate Smad4 tumor suppressive function. In this proposal, we propose to investigate how ALK-driven inactivation of Smad4 tumor suppressor contributes to TGF-β resistance in breast cancer. We hypothesize that ALK causes TGF-β resistance through Smad4 tyrosine phosphorylation and inactivation; thus, aberrant ALK activation in breast cells disrupts Smad4-exerted growth constraints to promote tumorigenesis. Consequently, suppression of ALK activity both restores Smad4 function and blocks other oncogenic activities of ALK, thus suppressing breast tumor formation.

Specifically, in this proposal, we will determine whether aberrant activation of ALK causes TGF-β resistance by Smad4 tyrosine phosphorylation and inactivation in breast cancer cell lines. Next, we will elucidate the molecular mechanism by which ALK-mediated Smad4 tyrosine phosphorylation affects Smad4 signaling. Finally, we will determine the impact of ALK activation on Smad4 Y95 phosphorylation, mammary tumor initiation, and progression, using human tissues and mouse models (including patient-derived xenografts).

BODY:

In our preliminary studies, we found that ALK can phosphorylate Smad4 on a particular tyrosine residue and inactivate Smad4 activity. During the first funding year of this proposal, we will investigate whether forced activation and knockdown of ALK affect TGF-β responses in breast cancer cell lines. We will also examine whether Smad4 Y95 phosphorylation disrupts TGF-β-induced cellular responses in breast cancer cell lines. In addition, we will determine if ALK-resistant Smad4 mutant restores the TGF-β responses in ALK-activated breast cells lines. We have completed our proposed work for year 2015-2016 as presented below:

Task 1. Generating a large panel of stable cell lines to determine the effect of Smad4 Y95 phosphorylation on TGF-β-induced cellular response.

As planned in our proposal, the major effort in our first funding year was to produce stable cell lines expressing caALK, knockdown of ALK or Smad4 or its variants in breast cancer cells. These cell lines will be our study system to facility our characterization of ALK and Smad4 phosphorylation.

Subtask 1. We used various cloning strategies such as PCR, restriction enzyme digestion et al., and have successfully generated expression constructs in lentiviral vectors encoding shALK (ALK-specific shRNA for ALK knockdown), caALK (a constitutively active form of ALK), kdALK (an kinase dead, inactive form of ALK by point mutation: K=>R at amino acid 210), Smad4, shSmad4 (Smad4-specific shRNA for Smad4 knockdown), Smad4-Y95E (a Y95 phosphorylation mimicking mutant of Smad4), and Smad-Y95F (a Y95 phosphorylation-resistant mutant of Smad4).

Subtask 2. We have transfected 293FT cells with these lentiviral constructs together with lenti virus packaging plasmids, and have produced lentiviral particles expressing shALK, shSmad4, caALK, ALK-K210R, Smad4, Smad4-Y95E, and Smad-Y95F.

Subtask 3. By infecting cells with these lentiviral particles and using puromycin drug as selecting marker, we have successfully generated the following stable cells:

- MDA-IBC3 cells that stably expressing shALK and shRNA control;
- MCF10A and MDA-MB-231 cells stably expressing caALK or ALK-K210R;
- Smad4-deficient MDA-MB-468, MCF10A, MDA-MB-231 and IBC3 cells stably expressing Smad4 WT, Smad4-Y95E, or Smad4-Y95F;
- More importantly, we have generated a Dox inducible expression of caALK or ALK-K210R under the control of tet-on promoter in MDA231 cells, hence, we control the intensity and duration for the expression of ectopic genes.

The successful establishment of these stable cells were confirmed by Western blot with specific antibodies and gene specific Q-PCR. Figure 1 is the representative data showing the successful establishment of stable cell lines.

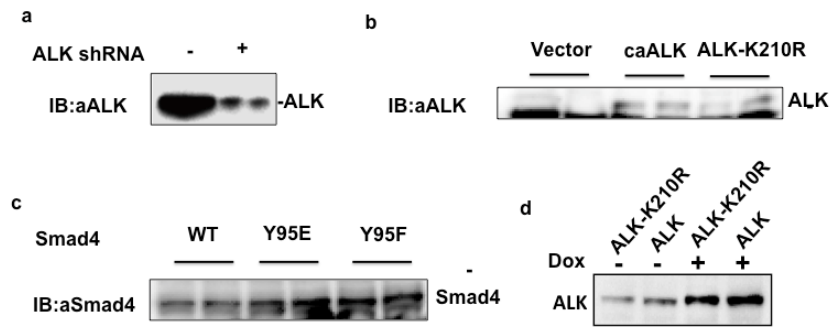

Figure 1. Establishment of ALK knockdown, ALK- expressing, or Smad4- expressing breast cancer stable cells. a, Stable knockdown of ALK by shALK in IBC3 cells. **b,** Stable expression of constitutively active caALK and kinase-dead ALK-K210R mutant in MDA231 cells. **c,** Stable expression of wild type Smad4 (WT), phosphorylation mimicking mutant of Smad4 (Y95E) and phosphorylation-resistant mutant of Smad4 (Y95F) in MDA468 cells. ALK was detected using anti-ALK antibody. Smad4 was detected using anti-Smad4 antibody. **d**, Dox inducible stable expression of ALK or ALK-K210R mutant in MDA231 cells.

Task 2. To examining the effect of ALK and Smad4 Y95 phosphorylation on the expression of TGF-β target genes such as cell cycle, extracellular matrix proteins, and EMT markers.

For this purpose, we used the stable cell lines generated above and carried out the following biochemical studies.

Subtask 1. We investigated the effect of ALK on TGF-β-induced target gene expression during TGF-β-induced cell growth inhibition by gain-of-function and loss-of-function approaches. Immortalized mammary epithelial cell line MCF10A stably expressing constitutively active form of ALK (caALK) or kinase-dead mutant of ALK (ALK-K210R), and ALK activity-high inflammatory breast cancer cell line IBC3 stably expressing control shRNA or shALK to knockdown ALK were used. Specifically, Q-PCR and Western blots were performed to examine the expression of cell cycle regulator p15, p21 and cyclin A. We found that in MCF10A cells TGF-β treatment increased the transcription of p15, p21 and cyclin A, and this induction was attenuated by caALK (NPM-ALK) expression while kinase-dead ALK (NPM-ALK-K210R) had no effect (Fig. 2a, 2b, and data not shown). IBC3 cells were not responsive to TGF-β treatment. However, knockdown of ALK sensitized cells to TGF-β-induced p15 and p21 mRNA level (Fig. 2c and 2d). Cell proliferation assay was also performed by MTS one these cell lines to reveal the effect of ALK on TGF-β-induced cell growth inhibition. We found that TGF-β treatment inhibited MCF10A cell growth, and this inhibition was lost in caALK (NPM-ALK)- expressing cells (Fig. 3a). Accordingly, treating cells with TAE684, a specific small molecule inhibitor of ALK, reversed this ALK anti-TGF-β, cell growth-promoting function (Fig. 3b). Furthermore, experiments with ALK knockdown cells showed that ALK depletion enhanced the expression of basal and TGF-β-induced level of cell cycle inhibitors such as p15 and p57 (Fig. 4a), and inhibited cell proliferation and sensitized cells to enhance TGF- β-induced growth inhibition (Fig. 4b).

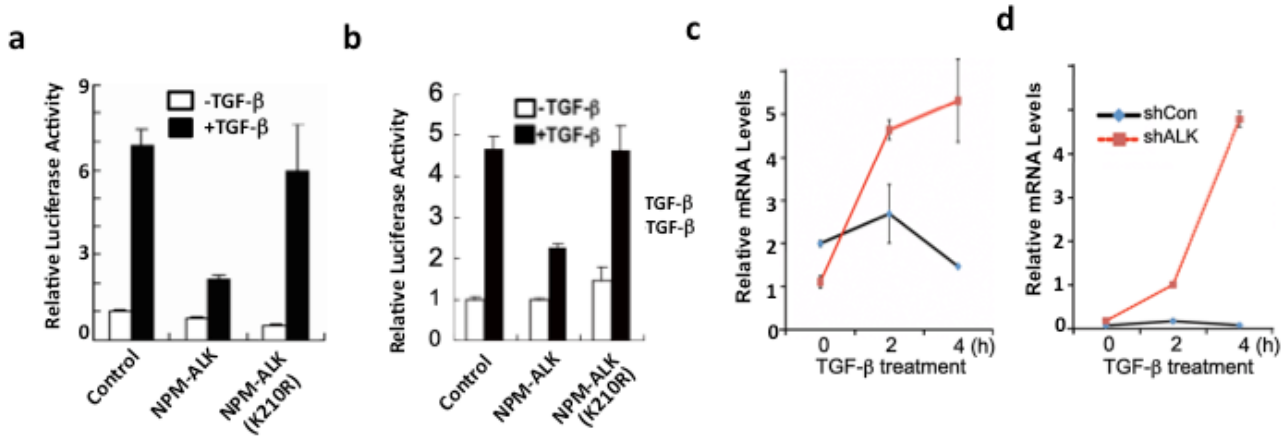

Figure 2. Effect of ALK on the expression of TGF-β target genes. Ectopic expression of active ALK (NPM-ALK) in MCF10A cells inhibited TGF-β induced p15 (**Fig. 2a**) and p21 (**Fig. 2b**) expression while the kinase-dead mutant of ALK (NPM-ALK (K210R)) lost its inhibitory effect. Knockdown of ALK in IBC3 cells restored TGF-β induced p15 (**Fig. 2c**) and p21 (**Fig. 2d**) expression. The transcription activity of p15 (**Fig. 2a**) or p21 (**Fig. 2b**) was determined by luciferase reporter assay under the control of endogenous p15 or p21 promoter. The mRNA level of p15 (**Fig. 2c**) or p21 (**Fig. 2d**) was determined by Q-PCR.

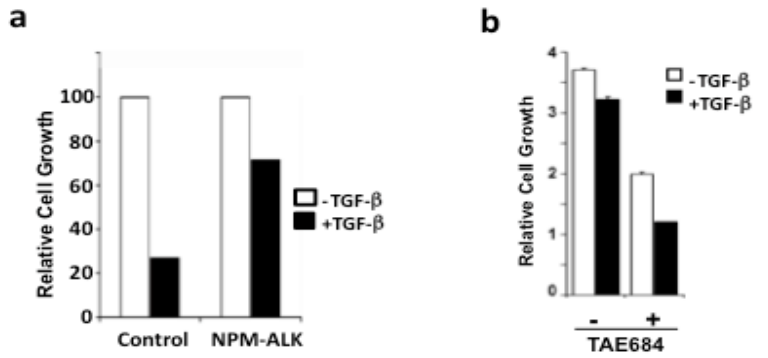

Figure 3. ALK abolishes the growth-inhibitory effect of TGF-β. TGF-β inhibited the proliferation of MCF10 cells while the expression of active ALK (NPM-ALK) promotes cell growth (**Fig. 3a)**. Treating cells with ALK-specific inhibitor TAE684 attenuated the stimulatory effect of ALK on cell proliferation (**Fig. 3b)**. Cell proliferation was determined by MTS assay.

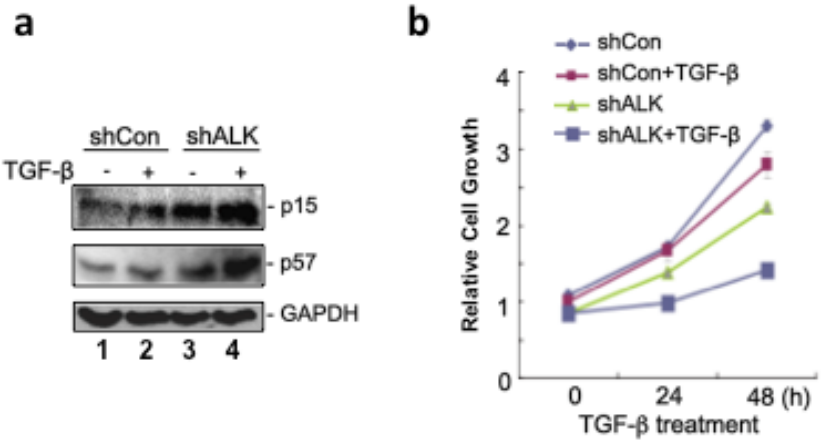

Figure 4. Loss of ALK restored TGF-β responses. Knockdown of ALK increased the expression of TGF-β target gene p15 and p57 in IBC3 cells (**Fig. 3a**). Knockdown of ALK restored the TGF-β-induced growth inhibition in IBC3 cells (**Fig. 3b**). The protein level of p15 and p57 was determined by IB with anti-p15- or anti- p57-specific antibody. Anti-GAPDH IB was used as loading control. Cell proliferation was measured by MTS assay.

Subtask 2. We next studied the effect of ALK on TGF-β-induced expression of extracellular matrix proteins such as PAI-1, MMP2 and MMP9, EMT markers such as Vimentin, β-Catenin, E-Catherin, N-Catherin, snail, cell migration and invasion in MDA-MB-231 cells stably expressing active ALK (NPM-ALK) or kinase-dead ALK (NPM-ALK-K210R), and in MDA-IBC3 cells that stably expressing shALK or shRNA control. We found that TGF-β induced the expression of extracellular matrix protein PAI-1, MMP2 and MMP9, while co-expression of active NPM-ALK attenuated this induction (Fig. 5a, 5b, and data not shown). Similarly, TGF-b induced the expression of EMT marker Snail, and this introduction was attenuated by co-expression of active ALK, but not by co-expression of ALK-K210R (Fig. 6 and data not shown). We further investigated the effect of ALK on cell migration and invasion by using Transwell assay. MDA-MB-231 cells were plated on Tranwell (with filter membrane for migration assay) or Transwell coated with extracellular matrix (for cell invasion assay) for overnight. Cells migrated through the membrane to the other side of well were stained with crystal violet for cell number counting. We found that TGF-β promoted MDA-MB-231 cell migration and invasion and this induction was abolished by ALK co-expression (Fig. 7).

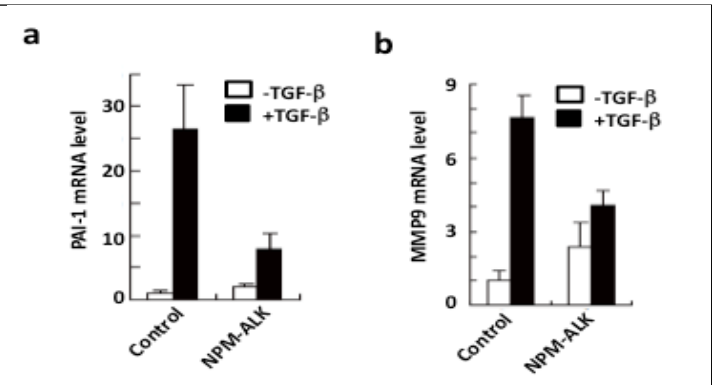

Figure 5. ALK inhibited TGF-β target gene expression. The TGF-β induced expression of PAI-1 (**Fig. 5a**) and MMP9 (**Fig. 5b)** was attenuated by caALK (NPM-ALK) co-expression. mRNA level of PAI-1 and MMP9 was determined by Q-PCR.

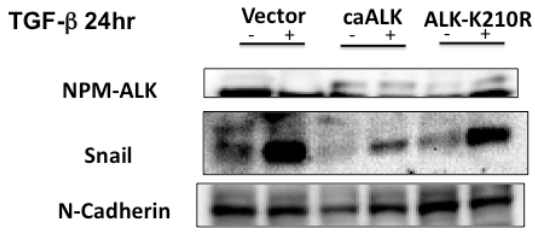

Figure 6. ALK inhibited TGF-β target gene expression. The expression of Snail and N-Cadherin was inhibited by caALK co-expression while kinase-dead mutant ALK-K210R had minimal effect. IB with anti- Snail- and N-Cadherin-specific antibodies was performed to examine their protein level.

Subtask 3. We investigated the functional importance of Smad4-Y95 phosphorylation by directly proving that Smad4-Y95 tyrosine phosphorylation mediates ALK suppression of TGF-β signaling. For this purpose, we used MDA-MB-468 cells that are Smad4 gene deletion and stably expressed WT Smad4 or phospho-mimicking Smad4-Y95E mutant. TGF-β-induced target gene expression such as the expression of cell cycle regulator was examined by reporter assay, Q-PCR and Western blots. TGF-β regulation on cell proliferation was also examined. We found that the expression of wild type Smad4 restored the TGF-β responses MDA-MB-468 cells as indicated by p15 promoter luciferase reporter assay (Fig. 8a). This TGF-β-induced p15 gene expression was inhibited by the co-expression of active ALK (NPM-ALK). However, Smad4-Y95E mutant failed to mediate TGF-β signaling, suggesting that Y95 phosphorylation leads to the loss of Smad4 activity (Fig. 8a). Similarly, reintroduction of wild type Smad4 restored the growth inhibitory effect of TGF-β in MDA-MB-468 cells, while Smad4-Y95E mutant was an inactive form of Smad4 (Fig. 8b).

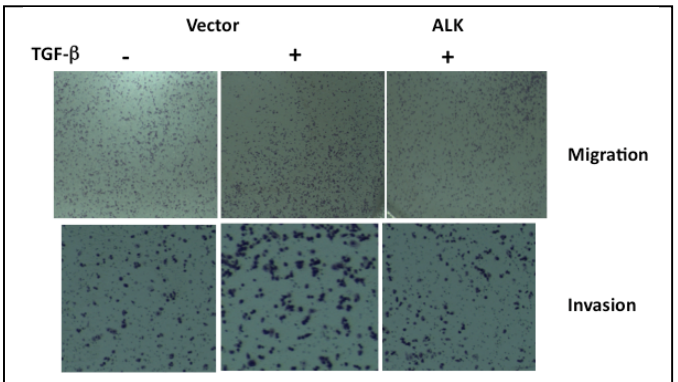

Figure 7. ALK inhibited TGF-β-induced cell migration and invasion. Migration and invasion assay was performed with Transwell.

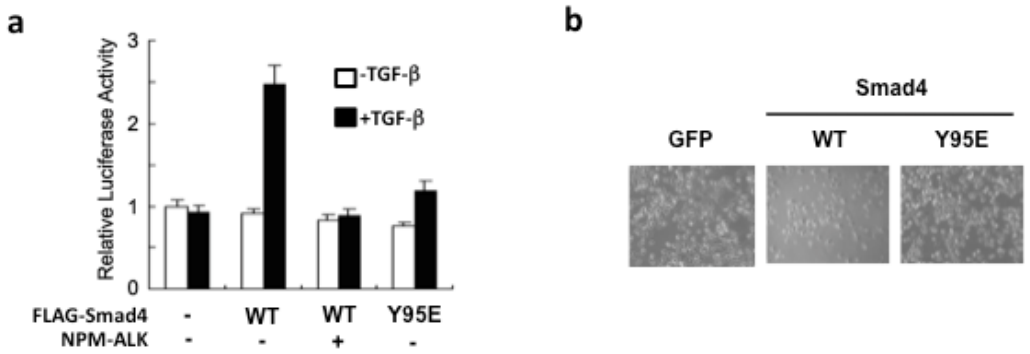

Figure 8. Tyrosine phosphorylation at Y95 of Smad4 inactivated Smad4 activity. a, p15 transcription was induced by TGF-β through Smad4-mediated signaling pathway, which could be repressed by NPM-ALK. However, the Y95 phosphorylation-mimicking form of Smad4-Y95E was unable to mediate TGF-β signaling. **b**. Wild type, but not Y95E mutant, could restore TGF-β growth inhibitory activity**.**

Subtask 4. We further investigated the functional importance of Smad4-Y95 phosphorylation by determining if ALK phosphorylation-resistant Smad4 mutant (Smad4-Y95F) restores the TGF-β responses in ALK-positive cells. We used Smad4-depleted MDA-IBC3 (MDA-IBC3-shSmad4) cells that are stably expressing wild type Smad4 or phosphorylation-resistant Smad4-Y95F mutant. Smad4-Y95F remains transcriptionally active, and cannot be phosphorylated by ALK (preliminary data), and presumably resist to the inhibitory effect of ALK on its activity. TGF-β-induced responses (e.g. gene transcription, growth inhibition and cell invasion/migration, as described above) were examined. Using p15 transcription assay measured by luciferase reporter activity as an example (Fig. 9), Smad4 could mediated TGF-β induction of p15 transcription, while phospho-mimic form of Smad4 (Y95E) was inactive in mediating this TGF-β response. However, we found that phosphorylation-resistant Smad4-Y95F mutant could mediate TGF-β responses and this response was not affected by NPM-ALK co-expression (Fig. 9), suggesting that ALK phosphorylation on Smad4 is essential for ALK regulation of TGF-β signaling activity. In supporting this, we found that Smad4-Y95F mutant was super-active in mediating TGF-β-regulated expression of EMT markers Twist, Snail and Vimentin (Fig. 10). Taken together, we determined that Y95 phosphorylation of Smad4 negatively regulates Smad4 activity and TGF-β signaling activity.

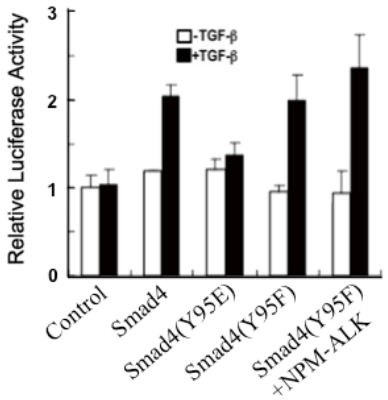

Figure 9. Phosphorylation-resistant Smad4(Y95F) is active and resistant to ALK activity. p15 transcription which was measured by luciferase activity was induced by Smad4-mediated TGF-β signaling. Phospho-mimic form of Smad4(Y95E) was inactive while Smad4(Y95F) was active and its activity was not affected by NPM-ALK co-expression.

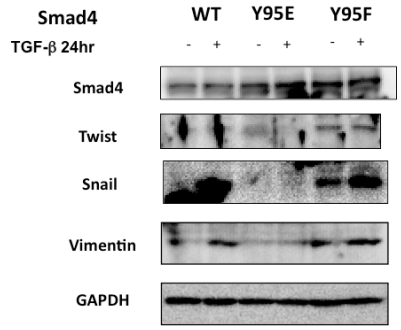

Figure 10. Phosphorylation-resistant Smad4(Y95F) is active in inducing the expression of EMT markers. The expression of Twist, Snail and Vimentin was induced by Smad4(Y95F) co-expression while Phospho-mimic form of Smad4(Y95E) was inactive. IB with anti- Twist-, Snail- and N-Cadherin-specific antibodies was performed to examine their protein level. IB with anti-GAPDH-specific antibody was used as loading control.

KEY RESEARCH ACCOMPLISHMENTS:

- Generated a large variety of ALK and Smad4 constructs and breast cancer stable cell lines that will be useful resources to share with other researchers.
- Determined that ALK inhibited TGF-β signaling in breast cancer cells.
- Determined that Smad4 Y95 phosphorylation inhibited TGF-β signaling in breast cancer cells.
- Determined that ALK inhibited TGF-β signaling though tyrosine phosphorylation of Smad4.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- TGF-β Meeting, Leiden, Netherlands, 8/21-23/2016. "Smad Signaling"
- 5th International Breast Cancer Stem Cell Symposium, Shanghai, 10/9-10/2016.
- icBEST-2016/isDDRHR – Chengdu, 10/14-17/2016. "Elucidating the mechanism underlying TGF-beta resistance in cancer"

CONCLUSION:

The oncogenic action of ALK has been believed to be through the signaling pathways (MEK/ERK, STAT3, PI3K/Akt), which promote cell proliferation while preventing cell death. Through our study, we for the first time revealed that ALK inhibited TGF-β signaling pathways. Furthermore, we found that ALK inhibited TGF-β signaling by tyrosine phosphorylating Smad4 at Y95. Therefore, our findings revealed a new aspect of ALK oncogenic functions, and decipher a novel crosstalk between ALK and TGF-β pathway in tumorigenesis.

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APPENDICES: N/A